Practitioner's Docket No. U013039-2

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PATENT TRADEMARK OFFICE

CHAPTER II

TRANSMITTAL LETTER TO THE UNITED STATES ELECTED OFFICE (EO/US)

(ENTRY INTO U.S. NATIONAL PHASE UNDER CHAPTER II)

PCT/ES99/00134	13 MAY 1999	13 MAY 1998
INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED

UTILIZATION OF INTERFERON ALPHA 5 IN THE TREATMENT OF VIRAL HEPATOPATHIES
TITLE OF INVENTION

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2. MA PILAR **CIVEIRA MURILLO**
3. ESTHER **LARREA LEOZ**

APPLICANT(S)

Box PCT
Assistant Commissioner for Patents
Washington D.C. 20231
ATTENTION: EO/US

CERTIFICATION UNDER 37 C.F.R. 1.10*
*(Express Mail label number is **mandatory**.)*
(Express Mail certification is optional.)

I hereby certify that this correspondence and the documents referred to as attached therein are being deposited with the United States Postal Service on this date NOVEMBER 1, 2000, in an envelope as "Express Mail Post Office to Addressee," Mailing Label Number EL699732521US, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

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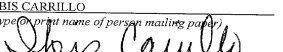

Signature of person mailing paper

WARNING: *Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.*

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*"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition." Notice of Oct. 24, 1996, 60 Fed Reg 56,439, at 56,442*

NOTE The completion of those filing requirements that can be made at a time later than 30 months from the priority date results from the Commissioner exercising his judgment under the authority granted under 35 USC 371(d). The filing receipt will show the actual date of receipt of the last item completing the entry into the national phase. See 37 C.F.R. §1.491 which states: "An international application enters the national state when the applicant has filed the documents and fees required by 35 USC 371(c) within the periods set forth in § 1.494 and § 1.495."

WARNING: *Where the items are those which can be submitted to complete the entry of the international application into the national phase are subsequent to 30 months from the priority date the application is still considered to be in the international state and if mailing procedures are utilized to obtain a date the express mail procedure of 37 C.F.R. §1.10 must be used (since international application papers are not covered by an ordinary certificate of mailing - See 37 C.F.R. §1.8.*

NOTE. Documents and fees must be clearly identified as a submission to enter the national state under 35 USC 371 otherwise the submission will be considered as being made under 35 USC 111. 37 C.F.R. § 1.494(f).

1. Applicant herewith submits to the United States Elected Office (EO/US) the following items under 35 U.S.C. 371:

   a. [X] This express request to immediately begin national examination procedures (35 U.S.C. 371(f)).

   b. [X] The U.S. National Fee (35 U.S.C. 371(c)(1)) and other fees (37 C.F.R. § 1.492) as indicated below:

2.Fees

CLAIMS FEE	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
[ ]*	TOTAL CLAIMS	2 - 20 =		x $ 18.00 =	$
	INDEPENDENT CLAIMS	1 - 3 =		x $ 80.00 =	
	MULTIPLE DEPENDENT CLAIM(S) (if applicable) + $270.00				
BASIC FEE**	[ ] U.S PTO WAS INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where an International preliminary examination fee as set forth in § 1.482 has been paid on the international application to the U.S. PTO: [ ] and the international preliminary examination report states that the criteria of novelty, inventive step (non-obviousness) and industrial activity, as defined in PCT Article 33(2) to (4) have been satisfied for all the claims presented in the application entering the national stage (37 CFR 1.492(a)(4)) ..... $100.00 [ ] and the above requirements are not met (37 CFR 1.492(a)(1)) ..... $690.00 [X] U.S. PTO WAS NOT INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where no international preliminary examination fee as set forth in § 1.482 has been paid to the U.S. PTO, and payment of an international search fee as set forth in § 1.445(a)(2) to the U.S. PTO: [ ] has been paid (37 CFR 1.492(a)(2)) ..... $710.00 [X] has not been paid (37 CFR 1.492(a)(3)) ..... $1,000.00 [ ] where a search report on the international application has been prepared by the European Patent Office or the Japanese Patent Office (37 CFR 1.492(a)(5)) $860.00				
			Total of above Calculations		-
SMALL ENTITY	Reduction by ½ for filing by small entity, if applicable. Affidavit must be filed. (note 37 CFR 1.9, 1.27, 1.28)				-
			Subtotal		500.00
			Total National Fee		$
	Fee for recording the enclosed assignment document $40.00 (37 CFR 1.21(h)). (See Item 13 below). See attached "ASSIGNMENT COVER SHEET".				
TOTAL			Total Fees enclosed		$ 500.00

*See attached Preliminary Amendment Reducing the Number of Claims.

i. [X] A check in the amount of $500.00 to cover the above fees is enclosed.
ii. [ ] Please charge Account No. _____ in the amount of $ _____.
A duplicate copy of this sheet is enclosed.

***WARNING:* *"To avoid abandonment of the application the applicant shall furnish to the United States Patent and Trademark Office not later than the expiration of 30 months from the priority date: * * * (2) the basic national fee (see § 1.492(a)). The 30-month time limit may not be extended." 37 C.F.R. § 1.495(b)*

WARNING. *If the translation of the international application and/or the oath or declaration have not been submitted by the applicant within thirty (30) months from the priority date, such requirements may be met within a time period set by the Office. 37 C.F.R. § 1.495(b)(2). The payment of the surcharge set forth in § 1.492(e) is required as a condition for accepting the oath or declaration later than thirty (30) months after the priority date. The payment of the processing fee set forth in § 1.492(f) is required for acceptance of an English translation later than thirty (30) months after the priority date. Failure to comply with these requirements will result in abandonment of the application. The provisions of § 1.136 apply to the period which is set. Notice of Jan. 3, 1993, 1147 O.G. 29 to 40.*

3. [X] A copy of the International application as filed (35 U.S.C. 371(c)(2)):

NOTE. *Section 1.495 (b) was amended to require that the basic national fee and a copy of the international application must be filed with the Office by 30 months from the priority date to avoid abandonment. "The International Bureau normally provides the copy of the international application to the Office in accordance with PCT Article 20. At the same time, the International Bureau notifies applicant of the communication to the Office. In accordance with PCT Rule 47.1, that notice shall be accepted by all designated offices as conclusive evidence that the communication has duly taken place. Thus, if the applicant desires to enter the national stage, the applicant normally need only check to be sure the notice from the International Bureau has been received and then pay the basic national fee by 30 months from the priority date." Notice of Jan. 7, 1993, 1147 O.G. 29 to 40, at 35-36. See item 14c below.*

a. [X] is transmitted herewith.
b. [ ] is not required, as the application was filed with the United States Receiving Office.
c. [ ] has been transmitted
i. [ ] by the International Bureau.
Date of mailing of the application (from form PCT/IB/308): _____.
ii. [ ] by applicant on _____.
Date

4. [X] A translation of the International application into the English language (35 U.S.C. 371(c)(2)):
a. [X] is transmitted herewith.
b. [ ] is not required as the application was filed in English.
c. [ ] was previously transmitted by applicant on _____.
Date
d. [ ] will follow.

5. [X] Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. 371(c)(3)):

NOTE *The Notice of January 7, 1993 points out that 37 C.F.R. § 1.495(a) was amended to clarify the existing and continuing practice that PCT Article 19 amendments must be submitted by 30 months from the priority date and this deadline may not be extended. The Notice further advises that: "The failure to do so will not result in loss of the subject matter of the PCT Article 19 amendments. Applicant may submit that subject matter in a preliminary amendment filed under section 1.121. In many cases, filing an amendment under section 1.121 is preferable since grammatical or idiomatic errors may be corrected." 1147 O.G. 29-40, at 36.*

a. [ ] are transmitted herewith.
b. [ ] have been transmitted
   i. [ ] by the International Bureau.
   Date of mailing of the amendment (from form PCT/IB/308): _____.
   ii. [ ] by applicant on _____.
   Date
c. [X] have not been transmitted as
   i. [X] applicant chose not to make amendments under PCT Article 19.
   Date of mailing of Search Report (from form PCT/ISA/210):
   AUGUST 13, 1999.
   ii. [ ] the time limit for the submission of amendments has not yet expired. The amendments or a statement that amendments have not been made will be transmitted before the expiration of the time limit under PCT Rule 46.1.

6. [X] A translation of the amendments to the claims under PCT Article 19 (38 U.S.C. 371(c)(3)):
a. [ ] is transmitted herewith.
b. [ ] is not required as the amendments were made in the English language.
c. [X] has not been transmitted for reasons indicated at point 5(c) above.

7. [X] A copy of the international examination report (PCT/IPEA/409)
[X] is transmitted herewith.
[ ] is not required as the application was filed with the United States Receiving Office.

8. [X] Annex(es) to the international preliminary examination report
a. [X] is/are transmitted herewith.
b. [ ] is/are not required as the application was filed with the United States Receiving Office.

9. [X] A translation of the annexes to the international preliminary examination report
a. [ ] is transmitted herewith.
b. [X] is not required as the annexes are in the English language.

10. [X] An oath or declaration of the inventor (35 U.S.C. 371(c)(4)) complying with 35 U.S.C. 115
    a. [ ] was previously submitted by applicant on _____. Date
    b. [X] is submitted herewith, and such oath or declaration
        i. [ ] is attached to the application.
        ii. [X] identifies the application and any amendments under PCT Article 19 that were transmitted as stated in points 3(b) or 3(c) and 5(b); and states that they were reviewed by the inventor as required by 37 C.F.R. 1.70.
    c. [ ] will follow.

Other document(s) or information included:

11. [X] An International Search Report (PCT/ISA/210) or Declaration under PCT Article 17(2)(a):
    a. [X] is transmitted herewith.
    b. [ ] has been transmitted by the International Bureau. Date of mailing (from form PCT/IB/308): _____.
    c. [ ] is not required, as the application was searched by the United States International Searching Authority.
    d. [ ] will be transmitted promptly upon request.
    e. [ ] has been submitted by applicant on _____. Date

12. [X] An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98:
    a. [ ] is transmitted herewith. Also transmitted herewith is/are:
        [ ] Form PTO-1449 (PTO/SB/08A and 08B).
        [ ] Copies of citations listed.
    b. [X] will be transmitted within THREE MONTHS of the date of submission of requirements under 35 U.S.C. 371(c).
    c. [ ] was previously submitted by applicant on _____. Date

13. [X] An assignment document is transmitted herewith for recording.

A separate [X] "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or [ ] FORM PTO 1595 is also attached.

_____
_____
_____

14. [X] Additional documents:
    a. [X] Copy of request (PCT/RO/101)
    b. [X] International Publication No. WO 99/58143
       i. [X] Specification, claims and drawing
       ii. [ ] Front page only
    c. [X] Preliminary amendment (37 C.F.R. § 1.121)
    d. [X] Other

FOR PCT/IB/308: DEMAND FORM PCT/IPEA/401: WRITTEN OPINION PCT/IPEA/2108: (2) FORM PCT/IPEA/416: (2) REPLY TO THE WRITTEN OPINIONS

15. [X] The above checked items are being transmitted
    a. [X] before 30 months from any claimed priority date.
    b. [ ] after 30 months.

16. [ ] Certain requirements under 35 U.S.C. 371 were previously submitted by the applicant on
_____, namely:

_____
_____
_____

AUTHORIZATION TO CHARGE ADDITIONAL FEES

WARNING: *Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges if extra claims are authorized*

NOTE: *"A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission Submission of the fee set forth in § 1 17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 C F.R. § 1 136(a)(3).*

NOTE: *"Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 C.F. R. § 1.26(a).*

[X] The Commissioner is hereby authorized to charge the following additional fees that may be required by this paper and during the entire pendency of this application to Account No. 12-0425___.

[X] 37 C.F.R. 1.492(a)(1), (2), (3), and (4) (filing fees)

WARNING: *Because failure to pay the national fee within 30 months without extension (37 C.F.R. § 1.495(b)(2)) results in abandonment of the application, it would be best to always check the above box.*

[ ] 37 C.F.R. 1.492(b), (c) and (d) (presentation of extra claims)

NOTE: *Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must*

only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 C.F.R § 1 492(d)), it might be best not to authorize the PTO to charge additional claim fees, except possible when dealing with amendments after final action.

[X] 37 C.F.R. 1.17 (application processing fees)
[X] 37 C.F.R. 1.17(a)(1)-(5)(extension fees pursuant to § 1.136(a).
[X] 37 C.F.R. 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. 1.311(b))

NOTE. Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 C.F.R § 1 311(b).

NOTE: 37 C F.R. 1.28(b) requires "Notification of any change in loss of entitlement to small entity status must be filed in the application . . prior to paying, or at the time of paying . . issue fee " From the wording of 37 C.F.R. § 1 28(b): (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.

[ ] 37 C.F.R. § 1.492(e) and (f) (surcharge fees for filing the declaration and/or filing an English translation of an International Application later than 30 months after the priority date).

SIGNATURE OF PRACTITIONER

Reg. No.: 25,858

WILLIAM R. EVANS
(type or print name of practitioner)

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PATENT
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Jesus Prieto VALTUENA, et al

Serial No.: 09/674,445 Group No.: --

Filed: November 1, 2000 Examiner: --

For: USE OF INTERFERON ALPHA 5 IN THE TREATMENT OF VIRAL LIVER DISEASES

Attorney Docket No.: U-013039-2

Commissioner Patents and Trademarks
Washington, DC 20231

PRELIMINARY AMENDMENT

Sir:

In response to the Official Action of May 4, 2001, please amend the application as follows:

IN THE SPECIFICATION:

Please rewrite the paragraphs beginning on page 9, line 26 and ending on page 11, line 18 as follows:

CERTIFICATE OF MAILING (37 CFR 1.8a)

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the United States Postal on the date shown below with sufficient postage as first class mail in an envelope addressed to the: Commissioner of Patents and Trademarks, Washington, DC 20231

CLIFFORD J. MASS
Type or print name of person mailing paper)

Date: July 5, 2001

(Signature of person mailing paper)

RNAm levels of IFNα and IFNβ were determined using a quantitative polymerase chain reaction reverse transcription (RT-PCR) method using a thermocycler (Perkin-Elmer Gene Amp PCR system 2400). Prior to reverse transcription 2 μg of total RNA (from both the liver and PBMC) were treated with 1 unit of deoxyribonuclease (DNAse I amplification grade, Gibco-BRL, Gaithersburg, MD, USA) to eliminate possible contaminating DNA. The presence of traces of DNA was checked by including control reactions without reverse transcription. This step is required because of the absence of introns in IFNα and IFNβ genes (18), which made it impossible for us to distinguish the product of PCR from the RNA or possible contaminating DNA. All the controls performed without reverse transcription were negative, indicating the absence of contaminating DNA. Total RNA was transcribed (60 minutes at 37°C) with 400 units of M-MuLV reverse transcriptase (Gibco- BRL, Gaithersburg, MD, USA) in a final volume of 40 μl of 5 x saline solution (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), supplemented with 5 mM DTT, 0.5 mM triphosphate dioxyribonucleotides (Boehringer Mannheim, Mannheim, Germany), 48 units of RNAsas inhibitor (Promega Corporation, MD, US) and 400 ng of random hexamers (Boehringer Mannheim, Mannheim, Germany). After denaturing the reverse transcriptase (95°C, 1 minute) and rapidly cooling over ice, a 10 μl aliquot (0.5 μg) of the cDNA was used to amplify the IFNα and IFNβ by PCR in 50 μl of 10 x PCR buffer (160 mM $(NH_4)SO_4$, 670 mM Tris-HCl pH 8.8, 0.1% Tween 20) supplemented with the direction and antidirection primers (40 ng of each one for IFNα and 60 ng for IFNβ), 1.2 mM $MgCl_2$ and 2 units of Biotaq™ DNA polymerase (Bioline, London, LTK). Control reactions without RNA were performed in all the experiments. As an internal control for each sample a fragment of β-actin cDNA was amplified using a 10 μl aliquot of the cDNA obtained previously. The IFNα was amplified by performing 30 or 33 cycles (PBMC or liver respectively) (94°C, 60°C

and 72°C during 20, 15 and 30 seconds for each step respectively), the INFβ was amplified by performing 30 or 35 cycles (PBMC or liver respectively) (94°C, 58°C and 72°C for 20, 15 and 30 seconds for each step respectively) and β-actin was amplified by reacting 18 or 25 cycles (PBMC or liver respectively) (94°C, 55°C and 72°C for 20, 15 and 30 seconds for each step respectively), protocols which avoid interference with the PCR reaction saturation stage. The oligonucleotides (5'-3') d(TCCATGAGATGATCCAGCAG) (SEQ ID NO:2) and d(ATTTCTGCTCTGACAACCTCCC) (SEQ ID NO:3) were used as direction and antidirection primers respectively to amplify a fragment of 274 pairs of bases located between nucleotides 240-514 in the human IFNα gene (19). These oligonucleotides are direction primers designed to amplify all the subtypes of IFNα. The oligonucleotides D(TCTAGCACTGGCTGGAATGAG) (SEQ ID NO:4) and d(GTTTCGGAGGTAACCTGTAAG) (SEQ ID NO:5) were the primers used to amplify a fragment of 276 base pairs located between nucleotides 349-625 of cDNA of human IFNβ (20) d(TCTACAATGAGCTGCGTGTG) (SEQ ID NO:6) and d(GGTGAGGATCTTCATGAGGT) (SEQ ID NO:7) were the primers used to amplify a fragment of 314 base pairs (nucleotides 1319-2079) of the β-actin gene (21).

Please rewrite the paragraph bridging pages 12 - 13 as follows:

The presence of C virus RNA in serum was determined using the RT-PCR technique (14, 22), using 2 pairs of specific primers for the non-coding 5' region of the C virus genome. The C virus RNA was quantified using the competitive PCR technique previously described by ourselves (22). The viral genotype was determined using Viazov's method (23) as already described previously (22, 24). The test 5'G(R)CCGTCTTGGGGCC(M)AAATGAT (SEQ ID

NO:8) was used to determine genotype 4.

Pages 27 - 29 delete in entirety and substitute the accompanying Sequence Listing:

USE OF INTERFERON ALPHA 5 IN THE TREATMENT OF VIRAL LIVER DISEASES

Scope of the invention

The invention relates to the production of interferon alpha 5 for use in compositions useful in the treatment of liver diseases of viral origin.

We have shown that IFN-alpha 5 is the sole subtype of alpha interferon produced in the healthy liver and that its levels are clearly reduced in chronic hepatitis C, which suggests that this substance may be of therapeutic value in the treatment of this disease and other forms of viral hepatitis. Knowing the coding gene sequence for this interferon, its production through recombinant DNA technology in different hosts makes it possible to develop effective drugs for the treatment of liver diseases of this type at their different stages of development.

State of the art

Infected cells can recognize the presence of a virus by sending out signals which result in the transcription and secretion of type I interferon (IFNα and IFNβ). IFNα is a family of thirteen polypeptides (subtypes) coded by different genes. IFNβ is a glycoprotein produced by a single gene. Different cell types produce both IFNα and IFNβ (1, 2).

Viral infection is the main stimulus for the production of type I interferon, although there are other factors which can increase its synthesis, such as bacterial components, double chain RNA, growth factors and other cytokines (1). In addition to having its antiviral effect, IFNα can

interact with certain cytokines and with T cells regulating the growth and differentiation of the cells in the immune system (3). IFNα genes are expressed as a matter of course in human tissue in healthy individuals (4), while the expression of particular subtypes is restricted to certain cell types (5, 6). The induction of IFN by viruses is mainly regulated at transcription level. The specific activation of transcription occurs through the interaction of cell factors induced by viruses with the domains regulating the promoters of IFNα genes (7).

All IFNα and IFNβ subtypes have a common receptor at the cell surface. Competitive binding tests at the receptor for different IFNα subtypes indicate that all of these combine at the same receptor, but with different affinities (8). The biological activity of the different subtypes of IFNα is little known. The IFNα 5 and IFNβ 8 interferon subtypes appear to be those having the greatest antiviral activity. Antiproliferative response also differs between the different subtypes (9). In humans unstimulated peripheral blood mononuclear cells express different IFNα subtypes (10).

A common mechanism for the persistence of viral infection is avoidance of the IFN system. Many viruses have developed strategies to avoid the antiviral effects of IFN. Specifically, a selective defect in the production of IFNα has been described in monocytes infected by human immunodeficiency virus (11).

Hepatitis C virus (HCV) is a single chain RNA virus which results in chronic infection in more than two thirds of persons infected. The prevalence of infection by HCV is

around 2 to 3% in the population of the West. Studies perfonned in Europe show that 33% of patients with chronic HCV infection develop cirrhosis in a mean period of less than 20 years (12). A significant proportion of these patients develop liver cancer, with an annual incidence of 1.4% (13). It has been difficult to find the reason for the high level of persistence of HCV infection. The high rate of mutations in the virus and the production of a predominant profile of Th2 cytokines in comparison with Th1 have been described as being responsible for this high level of persistence by the infection. Treatment with IFN induces a sustained response in around 30% of patients with chronic hepatitis C. The mechanism responsible for response or non-response to treatment with IFN is little understood.

The IFN system has only been studied in chronic HCV infection. There is no appropriate animal model for chronic HCV infection, and, because of this, investigations performed on humans are the only source of information on the pathophysiology and pathogenesis of chronic hepatitis C. This invention describes the expression of IFNα and IFNβ genes in the liver and in the peripheral blood mononuclear cells (PBMC) in healthy controls and patients with chronic hepatitis C. In addition to this we have analysed the IFNα subtype expressed in normal liver tissue and the liver tissue of patients with chronic hepatitis C. Expression of the different IFNα subtypes has also been analysed in PBMC in healthy controls and patients with chronic hepatitis C.

REFERENCES

1. Maeyer E, Maeyer-Guignard J. lnterferons. In Thomson A, ed. The Cytokine Handbook. London: Academic Press Limited 1991: 215-239.

2. Samuel CE. Antiviral Actions of Interferon. Interferon-Regulated Cellular Proteins and Their Surprisingly Selective Antiviral Activities. Virology 1991; 183: 1-11.

3. Tilg H. New Insights Into the Mechanisms of Interferon Alfa: An Immunoregulatory and Anti-inflammatory Cytokine. Gastroenterology 1997; 112: 1017-1021.

4. Tovey MG, Streuli M, Gresser I, Gugenheim I, Blanchard B, Guymarho J, Vignaux F and Gigou M. Interferon messenger RNA is produced constitutively in the organs of normal individuals. Proc. Natl. Acad. Sci. USA 1987; 84: 5038-5042.

5. Bisat F, Raj NB, Pitha PM. Differential and cell type specific expression of murine alpha interferon genes is regulated on the transcriptional level. Nucleic Acids Res 1988; 16:6067-6083.

6. Hiscott J, Cantell K, Weissmann C. Differential expression of human interferon genes. Nucleic Acids Res 1984; 12:3727-3746.

7. Au WC, Su Y, Raj NBK and Pitha PM. Virus-mediated Induction of Interferon A Gene Requires Cooperation between Multiple Binding Factors in the Interferon α Promoter

Region. The Journal of Biological Chemistry 1993, 268: 24032-24040.

8. Aguet M, Grobke M, Dreiding P. Various human interferon alpha subclasses cross-react with common receptors: their binding affinities correlate with their specific biological activities. Virology 1984;132:211-216.

9. Foster GR, Rodrigues 0, Ghouze F, Schulte-Frohlinde D, Testa D, Liao MJ, Stark GR, Leadbeater L, Thomas HC. Different relative activities of human cell derived interferon-alpha subtypes: interferon alpha 8 has very high antiviral potency. J Interferon and Cytokine Res. 1996; 16:1027-1033.

10. Brandt ER, Linnane AW, Devenish RJ. Expression of IFN A genes in subpopulations of peripheral blood cells. Br J Haematol 1994; 86:717-725.

11. Gendelman HE, Friedman RM, Joe S, Baca LM, Turpin JA, Dveksker G, Meltzer MS and Dieffenbach C. A Selective Defect of Interferon α Production in Human Immunodeficiency Virus-infected Monocytes. The Journal of Experimental Medicine 1990; 172: 1433-1442.

12. Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. Lancet 1997; 349:825-832.

13. Fattovich G, Giustina G, Degos F et al. Morbidity and Mortality in Compensated Cirrhosis Type C: A Retrospective

Follow-Up Study of 384 Patients. Gastroenterology 1997;112: 463-472.

14. Gil B, Qian Ch, Riezu-Boj JI, Civeira MP, Prieto J. Hepatic and extrahepatic HCV RNA strands in chronic hepatitis C: different patterns of response to interferon treatment. Hepatology 1993;18:1050-1054.

15. Lopez S, Reeves R, Island ML, Bandu MT, Christeff N, Doly J and Navarro S. Silencer Activity in the Interferon-A Gene Promoters. The Journal of Biological Chemistry 1997; 272: 22788-22799.

16. Knodell R, Ishak K, Black W, Chen T, Craig R, Kaplowitz N, Kiernan T, et al. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. Hepatology 1981; 1:431-435.

17. Chomczynsky P; Sacchi N. Single-step of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 1987; 162:156-159.

18. Weissmann C, Weber H. The interferon genes. Prog Nucleic Acid Res Mol Biol 1986; 33:251-300.

19. Goeddel DV, Leung DW, Dull TJ, Gross M, Lawn RM., McCandliss R, Seeburg PH, Ullrich A, Yelverton E, Gray PW. The structure of eight distinct cloned human leukocyte interferon cDNAs. Nature 1981; 290:20-26.

20. Derynck R, Content J, DeClercq E, Volckaert G, Tavernier J, Devos R, Fiers W. Isolation and structure of a human fibroblast interferon gene. Nature 1980; 285:542-547.

21. Ng SY, Gunning P, Eddy R, Ponte P, Leavitt J, Shows T, Kedes L. Evolution of the functional human b-actin gene and its multi-pseudogene family: conservation of noncoding regions and chromosomal dispersion of pseudogenes. Mol Cell Biol 1985; 5:2720-2732.

22. Larrea E, Garcia N, Qian Ch, et al. Tumor Necrosis Factor α Gene Expression And The Response To Interferon In Chronic Hepatitis C. Hepatology 1996; 23: 210-217.

23. Viazov S, Zibert A, Ramakrishnan K; Widell A; Cavicchini A, Schreier E; Roggendord M. Typing of hepatitis C virus isolates by DNA enzyme immunoassay. J. Virol. Methods 1994;48:81-92.

24. Sarobe P, Jauregui JI, Lasarte JJ, García N, Civeira MP, Borrás-Cuesta F and Prieto J. Production of interleukin-2 in response to synthetic peptides from hepatitis C virus E1 protein in patients with chronic hepatitis C: relationship with the response to interferon treatment. J Hepatol 1996;25:1-9.

DESCRIPTION OF THE INVENTION

Patients and controls

The expression of IFNα and IFNβ genes was analysed in samples from liver biopsies from 16 patients with chronic hepatitis C (9 men and 7 women, age range 24 to 71 years). Five of these patients showed cirrhosis. The viral genotype was determined in 14 patients and was 1b in 10 patients, 1a in 2 patients and genotype 3 in 1 patient.

In addition to this, expression of the IFNα and IFNβ genes was determined in 12 samples of normal liver obtained by laparotomy from 12 control patients (9 men and 3 women, age range 49 to 70 years). The laparotomies were performed on account of the presence of digestive tumours in 10 patients (4 colo-rectal, 5 gastric and 1 pancreatic) due to chronic pancreatitis in 1 patient and the presence of a hydatid cyst in another patient. Liver histology was normal in the twelve cases. None of these control cases had received treatment before the liver sample was obtained.

RNAm levels of IFNα and IFNβ were also determined in PBMC in 25 patients with chronic hepatitis C (14 men and 11 women, age range 24 to 69 years) (four of these patients had cirrhosis) and in PBMC from 23 healthy controls (10 men and 13 women, age range from 25 to 66 years). The viral genotype for these patients was 1b in 22 patients, 1a in two patients and 3 in 1 patient.

The diagnosis of chronic hepatitis C was based on an increase in serum transaminases lasting more than 6 months, a positive result for anti-HCV antibodies (2nd generation ELISA, Ortho Diagnostic System, Raritan, NJ, USA), the presence of C virus RNA in serum (reverse-reaction transcription in the polymerase chain), and histological evidence of chronic hepatitis. The severity of liver damage was evaluated using the Knodell index (16). Other causes of chronic hepatitis other than hepatitis C virus were ruled out. None of the patients had received treatment with IFNα during at least 6 months prior to the study.

Preparation of liver, PBMC and serum samples

The liver samples were obtained by liver biopsy using a Tru-Cut biopsy needle (Baxter, Deerfield, IL). One third of the sample was immediately frozen in liquid nitrogen and kept at -80°C until total RNA extraction took place. The remainder of the sample was used for the histological investigation.

PBMC were isolated from heparinized blood using a density gradient with Lymphoprep (Nycomed Pharma As, Oslo, Norway), centrifuged at 600 g for 30 minutes. After centrifuging the PBMC were collected, washed 5 times with 0.9% NaCl and lysed using Ultraspec™ protein denaturing solution (Biotech Laboratories, Houston, USA). The cellular lysate was kept at -80°C until total RNA extraction was performed using the method of Chomcznski and Sacchi (17).

The serum samples were obtained by centrifuging from venous blood collected in sterile tubes. The serum was kept at -40°C until use.

Analysis of the expression of IFNα and IFNβ genes in the liver and PBMC

RNAm levels of IFNα and IFNβ were determined using a quantitative polymerase chain reaction reverse transcription (RT-PCR) method using a thermocycler (Perkin-Elmer Gene Amp PCR system 2400). Prior to reverse transcription 2 µg of total RNA (from both the liver and PBMC) were treated with 1 unit of deoxyribonuclease (DNAse I amplification grade, Gibco-BRL, Gaithersburg, MD, USA) to eliminate possible contaminating DNA. The presence of

traces of DNA was checked by including control reactions without reverse transcription. This step is required because of the absence of introns in IFNα and IFNβ genes (18), which made it impossible for us to distinguish the product of PCR from the RNA or possible contaminating DNA. All the controls performed without reverse transcription were negative, indicating the absence of contaminating DNA. Total RNA was transcribed (60 minutes at 37°C) with 400 units of M-MuLV reverse transcriptase (Gibco- BRL, Gaithersburg, MD, USA) in a final volume of 40 µl of 5 x saline solution (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), supplemented with 5 mM DTT, 0.5 mM triphosphate dioxyribonucleotides (Boehringer Mannheim, Mannheim, Germany), 48 units of RNAsas inhibitor (Promega Corporation, MD, US) and 400 ng of random hexamers (Boehringer Mannheim, Mannheim, Germany). After denaturing the reverse transcriptase (95°C, 1 minute) and rapidly cooling over ice, a 10 µl aliquot (0.5 µg) of the cDNA was used to amplify the IFNα and IFNβ by PCR in 50 µl of 10 x PCR buffer (160 mM $(NH_4)SO_4$, 670 mM Tris-HCl pH 8.8, 0.1% Tween 20) supplemented with the direction and antidirection primers (40 ng of each one for IFNα and 60 ng for IFNβ), 1.2 mM $MgCl_2$ and 2 units of Biotaq™ DNA polymerase (Bioline, London, LTK). Control reactions without RNA were performed in all the experiments. As an internal control for each sample a fragment of β-actin cDNA was amplified using a 10 µl aliquot of the cDNA obtained previously. The IFNα was amplified by performing 30 or 33 cycles (PBMC or liver respectively) (94°C, 60°C and 72°C during 20, 15 and 30 seconds for each step respectively), the INFβ was amplified by performing 30 or 35 cycles (PBMC or liver respectively) (94°C, 58°C and 72°C for 20, 15 and 30

seconds for each step respectively) and β-actin was amplified by reacting 18 or 25 cycles (PBMC or liver respectively) (94°C, 55°C and 72°C for 20, 15 and 30 seconds for each step respectively), protocols which avoid interference with the PCR reaction saturation stage. The oligonucleotides (5'-3') d(TCCATGAGATGATCCAGCAG) and d(ATTTCTGCTCTGACAACCTCCC) were used as direction and antidirection primers respectively to amplify a fragment of 274 pairs of bases located between nucleotides 240-514 in the human IFNα gene (19). These oligonucleotides are direction primers designed to amplify all the subtypes of IFNα. The oligonucleotides d(TCTAGCACTGGCTGGAATGAG) and d(GTTTCGGAGGTAACCTGTAAG) were the primers used to amplify a fragment of 276 base pairs located between nucleotides 349-625 of cDNA of human IFNβ (20). d(TCTACAATGAGCTGCGTGTG) and d(GGTGAGGATCTTCATGAGGT) were the primers used to amplify a fragment of 314 base pairs (nucleotides 1319-2079) of the β-actin gene (21).

After the amplification reactions 20 µl of the PCR product were run in a 2% agarose gel containing ethidium bromide. The bands obtained were displayed using an ultraviolet lamp and were analysed using a commercial programme (Molecular Analyst/PC, Bio-Rad) capable of digitizing and analysing the image obtained. Finally the values corresponding to the expression of the IFNα and IFNβ genes were standardized with their β-actin correlates. The results are expressed as the quotient between the value of IFNα and IFNβ and the β-actin correlate. Previously we demonstrated that the RNAm of β-actin was expressed constantly both in the liver and in the PBMC of patients with chronic hepatitis C (22),

which has enabled us to standardize IFNα and IFNβ values with those obtained for β-actin.

Validation curves for the PCR technique were prepared using known quantities of total RNA (from 0 up to 1 μg). As will be seen in Figure 3, with the total initial RNA quantities used for IFNα, IFNβ and β-actin (0.5 μg, for both the liver and PBMC), we were within the linear range of the PCR amplification curve. The inter-test coefficient of variance for IFNα/β-actin was 22% and for IFNβ/β-actin it was 24%. The identity of the PCR product obtained was checked for IFNα and IFNβ by automatic sequencing (ABI prism™ 310 genetic analyser, Perkin Elmer).

Identification of IFNα subtypes

Total RNA extraction, reverse transcription and the PCR reaction were performed as described above, using the IFNα direction primers mentioned. The PCR product obtained was cloned using the commercial TOPO TA cloning kit (Invitrogen, Leek, Holland). At least 6 clones from each insert were sequenced in an automatic ABI PRISM 310 sequencer (Perkin Elmer, Foster, CA), using the Dye Rhodamine Terminator Cycle Sequencing Kit (Perkin Elmer, Foster, CA).

Detection, quantification and genotyping of C virus RNA

The presence of C virus RNA in serum was determined using the RT-PCR technique (14, 22), using 2 pairs of specific primers for the non-coding 5' region of the C virus genome. The C virus RNA was quantified using the competitive PCR technique previously described by ourselves (22). The viral

genotype was determined using Viazov's method (23) as already described previously (22, 24). The test 5'G(A,G)CCGTCTTGGGGCC(A,C)AAATGAT was used to determine genotype 4.

Statistical analysis

The IFNα and IFNβ results are presented as mean ± standard error. The normality of the variables was studied using the Shapiro-Wilks test. Statistical analysis of IFNα and IFNβ values in PBMC or liver was performed using non-parametric tests (Mann-Whitney U test) or parametric tests (Student's T). The association between quantitative variables was investigated using the Pearson or Spearman correlation coefficient, as appropriate. Windows SPSS 6.0 program was used for the statistical analysis.

Production of recombinant protein

Expression and purification of human interferon-α5 in *Escherichia coli*:

Despite the fact that the expression of cDNAs originating from eucaryote organisms in *Escherichia coli* in general ensures a high level of production, isolation and purification of the protein of interest involves complex procedures and low yields. For this reason expression vectors are used to help obtain merged proteins whose purification is reduced to an affinity chromatography step, with high yield and efficiency.

Construction of the expression vector and acquisition of recombinant bacteria

The cDNA which codes for interferon-α5 is cloned in pET14b vector (available commercially from Novagen). This vector

provides a sequence which codes for a series of histidine residues (1 kDa) which are translated in phase with the cloned cDNA to yield a merged protein which includes a 1 kDa histidine tail at its terminal amine end and then interferon-α5, with a site between the two which can be cut by thrombin.

Once the expression vector has been obtained, competent bacteria of the BL21 (DE3) strain are prepared, as this strain contains a gene which can be induced by T7 RNA polymerase, which is a necessary requirement for the subsequent production of protein. The competent bacteria are converted with the vector previously obtained (pET14b with the cloned interferon-α5 cDNA). The transformed bacteria are selected by their growth in LB medium with ampicillin, as the vector contains a gene which is resistant to this antibiotic.

Expression and purification of interferon-α5:

The transformed bacteria are grown in LB medium with ampicillin at 37°C until an optical density of 0.4 at 600 nm is obtained. Then expression of the recombinant protein with IPTG is induced at a final concentration of 0.5 mM. In this way the *lac* promoter is induced and as a consequence the T7 RNA polymerase prometer which contains the vector and which regulates the expression of the cloned cDNA is induced. The culture is grown for a further 4 hours under the same conditions.

To obtain the extracts, once the bacteria have grown, centrifuging is carried out at 4°C. The precipitated bacteria are resuspended in 10 mM Tris/HCl buffer, 10%

saccharose, 2 mM 2-mercaptoethanol and protease inhibitors. Homogenization was performed ultrasonically by incubation for 30 minutes with lysozyme at 4°C. This breaks down the bacterial wall and improves the yield of the extraction process. The cytosol extract is obtained by centrifuging the homogenate at 100,000 g for 90 minutes. Protein production is checked by analysing the cytosol fraction by SDS-PAGE.

His-interferon-α5 merged protein is purified by chromatography of the cytosol extract in a 2 ml nickel column. The protein is eluted by washing the column with 1 M imidazole. The pure protein is processed with thrombin and the interferon-α5 is subsequently repurified by molecular exclusion chromatography.

Expression and purification of human interferon-α5 in *Solanum tuberosum*:

Construction of the expression vector and acquisition of transgenic plants.

The cDNA which codes for interferon-α5 is cloned in an *Agrobacterium tumefaciens* expression vector. This vector contains the potato promoter (the most abundant protein in the *Solanum tuberosum* tubercle), as well as a sequence which codes for a series of histidine residues (1 kDa) and which are translated in phase with the cloned cDNA to yield a merged protein which contains a 1 kDa histidine tail at its terminal amine end followed by interferon-α5, with a site between the two which can be cut by thrombin.

Once the expression vector has heen obtained, competent bacteria of the GV2260 strain of *Agrobacterium tumefaciens*

are prepared. The competent bacteria are transformad using the previously obtained vector. The transformed bacteria are selected by growth in LB medium with kanamycin, as the vector contains a gene which is resistant to that antibiotic.

Subsequently a coculture of the transformed bacteria with the plant material (*Solanum tubersosum* leaves cultivated *in vitro*) is performed and the plant cells resistant to kanamycin are selected. These cells are regenerated until transgenic plants are obtained.

Acquisition and purification of interferon-α5:

Total protein extraction is performed from tubercles of the transgenic plants which express the interferon-α5.

The purification of His-interferon-α5 merged protein is carried out by chromatography of the protein extract obtained on a 2 ml nickel column. The protein is eluted by washing the column with 1 M imidazole. The pure protein is processed with thrombin and the interferon-α5 is subsequently repurified using molecular exclusion chromatography.

IFNα subtypes in normal liver tissue and PBMC in healthy individuals

After extraction of the total RNA of the normal liver tissue samples the RNAm of the IFNα was amplified using universal primers for all the IFNα subtypes. The PCR amplification products were then cloned and sequenced. 41 clones from 4 different normal livers were analysed and we observed that the IFNα sequence in the 41 clones was the same and corresponded to the IFNα5 subtype (Table 1). These

results show that IFNα5 is the only IFNα subtype expressed in normal liver. The partial cDNA sequence of the IFNα5 obtained from all the clones was shown to be SEQ ID NO: 1.

To compare the profile of the IFN subtypes expressed in the liver with that expressed in PBMC the total RNA of the PBMC from 5 healthy controls was extracted and the IFNα RNAm was amplified with the universal primers for all the IFNα subtypes. Of the 43 clones analysed, 15 corresponded to the IFNα5 subtype, 14 to the IFNαl/13, 6 to the IFNα21 and 8 clones to other IFNα subtypes (Table 1). These results indicate that the IFNα subtype profile expressed in PBMC differs from that expressed in normal liver.

IFNα subtypes in liver tissue and PBMC from patients with chronic hepatitis C

The above results show that the normal liver expresses IFNα5, while PBMC express a variety of IFNα subtypes. In the liver parenchyma of patients with chronic hepatitis C there is mononuclear cell infiltrate, an important source of IFNα. This suggests that the profile of IFNα subtypes expressed by the liver in patients with chronic hepatitis C might differ from the profile found in normal liver. To investigate the expression of IFNα subtypes in chronic hepatitis C we extracted the total RNA from liver samples from 3 different patients and 2 PBMC samples. After amplifying the IFNα RNAm with universal primers for all subtypes, we cloned and sequenced 24 clones of liver tissue and 18 clones of PBMC. As shown in Table 1, the PBMC from patients with chronic hepatitis C expressed IFNα21, IFNα5

and IFNα7 (5, 12, and 1 clones respectively). In the liver tissue from these patients we found subtypes IFNα21, IFNα17 and IFNα1/13 (8, 1 and 2 clones respectively) in addition to the IFNα5 subtype (Table 1).

These data suggest that the production of IFNα by the mononuclear cell infiltrate can cause a change in the profile of IFNα subtypes expressed in the liver tissue of patients with chronic hepatitis C.

Levels of expression of IFNα RNAm in PBMC and the liver of patients with chronic hepatitis C and controls

Total RNA was extracted from PBMC and liver samples from patients with chronic hepatitis C (n=25 and 16, respectively), PBMC samples from healthy controls (n=20) and normal liver tissue samples obtained by laparotomy (n=12). The RNAm levels of IFNα were determined using the semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) technique using universal primers to amplify all the IFNα subtypes. The values are expressed as the ratio of IFNα RNAm to β-actin RNAm.

We found that the levels of expression of IFNα in the PMBC of patients with chronic hepatitis C were significantly increased in comparison with those found in healthy controls (3.2 ± 0.48 against 1.14 ± 0.26; p=0.001) (Figure 1A). This result was expected in a viral infection such as hepatitis C in which the PBMC are infected (14). On the other hand the levels of expression of IFNα RNAm were significantly reduced in the liver tissue from patients with chronic hepatitis C in comparison with that expressed

in normal liver (0.12 ± 0.03 against 0.43 ± 0.12; p=0.003) (Figure 1B).

As observed previously, IFNα5 is the only IFNα subtype detected in normal liver, while a mixture of subtypes is observed in the liver tissue of patients with chronic hepatitis C. Our findings indicate that in infection by HCV there is a marked reduction in the expression of the IFNα subtype normally expressed in liver tissue. Interestingly, IFNα RNAm levels in the livers of patients with chronic hepatitis C show a direct correlation with the Knodell index (r=0.54; p<0.05). This finding, together with the observation that the IFNα subtypes detected in the livers of patients with chronic hepatitis C are those observed in PBMC suggests that most of the IFNα RNAm found in the liver in hepatitis C comes from the inflammatory infiltrate. It appears possible that the reduction in the expression of liver IFNα (IFNα5) may play a part in making the HCV infection chronic. As a result, these observations may have therapeutic implications if we also bear in mind the marked antiviral and antiproliferative activity of the IFNα5 described by other authors (9).

Levels of expression of IFN RNAm in the PBMC and liver of patients with chronic hepatitis C and controls

IFNβ, the second majority form of type 1 interferon, is a glycoprotein produced by a single gene. In viral infections transcription of the IFNα and IFNβ genes is activated or repressed by various mechanisms (15). To analyse the expression of IFNβ in chronic hepatitis C we determined IFNβ RNAm levels in the same samples of liver tissue and PBMC previously used to determine the expression of IFNα.

As shown in Figure 2, we observed that IFNβ RNAm levels (expressed as a ratio against β-actin) were significantly higher in both PBMC and the liver in patients with chronic hepatitis C in comparison with the PBMC findings in healthy controls and normal livers (1.66 ± 0.2 against 0.88 ± 0.16; p=0.008 in PBMC and 1.37 ± 0.23 against 0.97 ± 0.16; p=0.011 in liver). These results show that while HCV causes IFNα to be repressed in the liver, the expression of IFNβ is increased in both the liver and PBMC. This indicates that VHC modulates the different type I IFN genes in the liver in a different way, and blocks the production of IFNα to permit the overexpression of IFNβ.

Relationship between the expression of IFNα and IFNβ genes with viral load, genotype and liver damage in chronic hepatitis C

In order to determine whether the expression of the IFNα or IFNβ genes can be related to viral load or genotype we quantified the C virus RNA in the serum of all patients using the competitive PCR technique and determined the VHC genotype using a hybridization method with specific test materials. We found no correlation between the expression of the IFNα or IFNβ genes (in the liver or PBMC) and C virus RNA levels in serum or the viral genotype.

Analysing the relationship between the expression of the type I IFN genes and the severity of liver damage in patients with chronic hepatitis C we found that IFNβ RNAM levels in the liver correlated directly with serum aspartate aminotransferase values (r=0.64, p=0.008) and the Knodell index (r=0.66, p=0.006). Likewise the IFNα RNAm

values in the liver showed a direct positive correlation with the Knodell index as mentioned previously.

Table 1. IFNα subtypes in controls and patients with chronic hepatitis C.

	Liver	PBMC
Control 1	9 IFNA5 clones	
Control 2	9 IFNA5 clones	
Control 3	11 IFNA5 clones	
Control 4	12 IFNA5 clones	
Control 5		3 IFNA5 clones 4 IFNA21 clones 2 IFNA1 clones
Control 6		8 IFNA5 clones
Control 7		10 IFNA1/13 clones 1 IFNA8 clone
Control 8		3 IFNA5 clones 2 IFNA21 clones 2 IFNA1/13 clones 1 IFNA22 clones
Control 9		2 IFNA10 clones 1 IFNA5 clone 1 IFNA2 clone 1 IFNA7 clone 1 IFNA8 clone 1 IFNA4 clone
RNA-VHC (+) 1	6 IFNA5 clones 2 IFNA21 clones 1 IFNA17 clone	7 IFNA5 clones 1 IFNA21 clone 1 IFNA7 clone
RNA-VHC (+) 2	2 IFNA5 clones 4 IFNA21 clones	5 IFNA5 clones 4 IFNA21 clones
RNA-VHC (+) 3	5 IFNA5 clones 2 IFNA21 clones 2 IFNA1 clones	

Description of the figures

Figure 1: Expression of alpha interferon/β-actin RNAm (ordinate) in peripheral blood mononuclear cells (A) and in the liver (B) of healthy controls and patients with chronic hepatitis C (HCV-RNA+) (abscissa).

Figure 2: Expression of beta interferon/β-actin RNAm (ordinate) in peripheral blood mononuclear cells (A) and in the liver (B) of healthy controls (C) and patients with chronic hepatitis C (HCV-RNA+) (abscissa).

Figure 3: Relationship between the initial quantity of total RNA (abscissa) and the strength of the PCR product band obtained by amplifying the RNAm of IFNα (•), IFNβ (▲) and β-actin (◆) (ordinate, as counts x mm^2) in PBMC (A) and liver (B) samples.

CLAIMS

1.- Use of IFN-alpha 5 or the gene sequence coding for it and/or essentially derived gene sequences for the manufacture of compositions useful in the treatment of liver diseases.

2.- Use according to claim 1, for the manufacture of compositions useful in the treatment of chronic hepatitis C.

3.- Use according to claim 1, for the manufacture of compositions useful in the treatment of cirrhosis of viral origin.

4.- Use according to claim 1, for the manufacture of compositions useful in the treatment of hepatocellular carcinoma.

5.- Use according to any one of claims 1-4, in which the manufactured composition is used to genetically induce physiological synthesis of interferon alpha 5, at nuclear level, in diseased liver cells deficient in that synthesis.

6.- Use according to any one of claims 1-4, in which manufacture of the composition comprises developing a recombinant protein for human application by cloning an expression vector in an appropriate host.

7.- Use according to claim 6, in which the cloned host is a eucaryote organism, preferably *Escherichia Coli*.

8.- Use according to claim 6, in which the cloned host is a procaryote organism, preferably *Solanum tuberosum*.

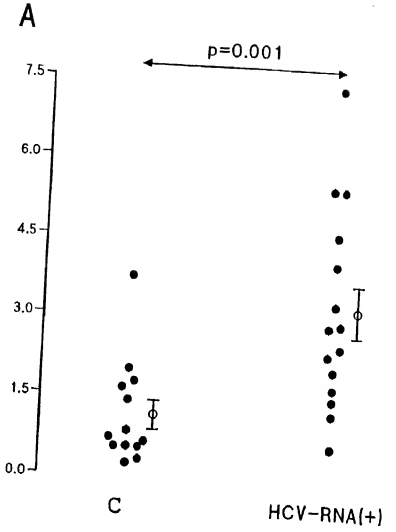
9.- Use according to any one of the foregoing claims, in which the manufactured composition is a composition which can be ingested with food.

10.- Use according to claims 1 to 4, characterised in that the manufactured composition is a composition for somatic gene therapy.

ABSTRACT

UTILIZATION OF INTERFERON ALPHA 5 IN THE TREATMENT OF VIRAL HEPATOPATHIES.

The invention relates to the use of interferon alpha 5 in the treatment of viral hepatopathies. The invention describes the reduced synthesis of IFNα5 in the livers of patients with hepatitis C in comparison to healthy livers. The sub-type of IFN expressed in said healthy livers corresponded only to the subtype alpha 5 in comparison with the different sub-types expressed in ill livers. The sequence SEQ ID NO:1 shows the partial sequence of cDNA corresponding to IFNα5. These significant differences between the expression patterns of some livers an others demonstrate the importance of the use of such interferon sub-type in the fabrication of compositions useful in the treatment of viral hepatopathies. The invention discloses in details such utilization in different forms and processes, including those which use the production of recombinant proteins from sequences of the type SEQ ID NO:1.



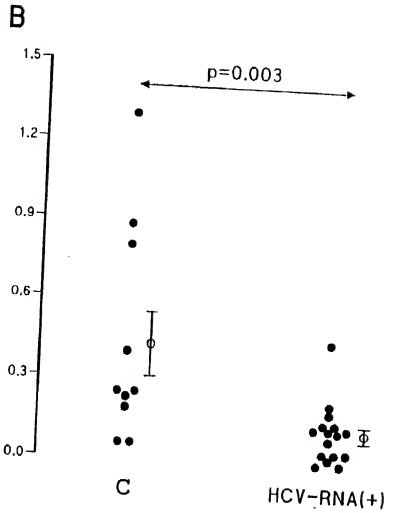


Figure 1

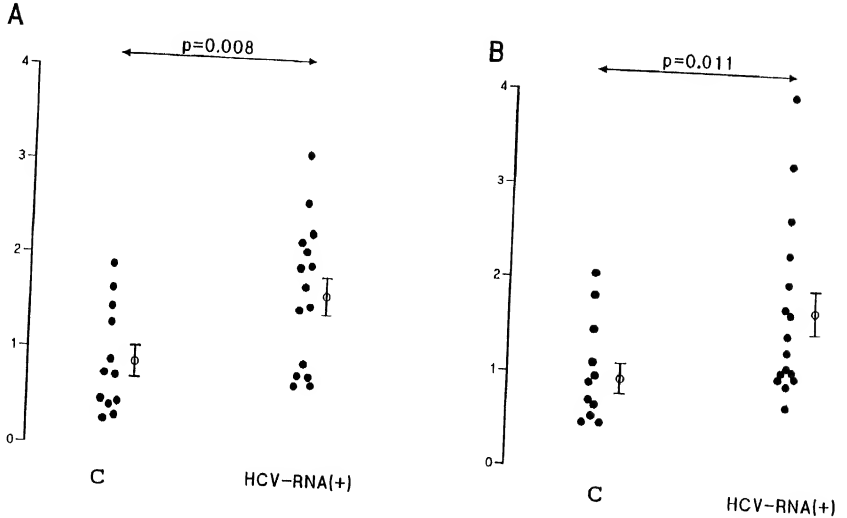


Figure 2

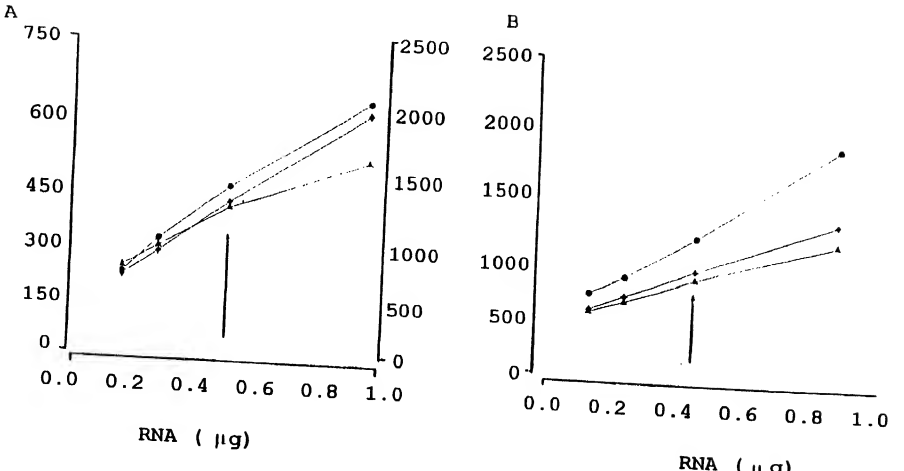


Figure 3

Practitioner's Docket No. U013039-2 *PATENT*

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COMBINED DECLARATION AND POWER OF ATTORNEY

(ORIGINAL, DESIGN, NATIONAL STAGE OF PCT, SUPPLEMENTAL, DIVISIONAL, CONTINUATION, OR C-I-P)

As a below named inventor, I hereby declare that:

TYPE OF DECLARATION

This declaration is of the following type:

(check one applicable item below)

[ ] original.
[ ] design.

NOTE: With the exception of a supplemental oath or declaration submitted in a reissue, a supplemental oath or declaration is not treated as an amendment under 37 CFR 1.312 (Amendments after allowance). M.P.E.P. Section 714.16, 7th Ed.

[ ] supplemental.

NOTE: If the declaration is for an International Application being filed as a divisional, continuation or continuation-in-part application, do not check next item; check appropriate one of last three items.

[X] national stage of PCT.

NOTE: If one of the following 3 items apply, then complete and also attach ADDED PAGES FOR DIVISIONAL, CONTINUATION OR C-I-P.

NOTE: See 37 C.F.R. Section 1.63(d) (continued prosecution application) for use of a prior nonprovisional application declaration in the continuation or divisional application being filed on behalf of the same or fewer of the inventors named in the prior application.

[ ] divisional.
[ ] continuation.

NOTE: Where an application discloses and claims subject matter not disclosed in the prior application, or a continuation or divisional application names an inventor not named in the prior application, a continuation-in-part application must be filed under 37 C.F.R. Section 1.53(b) (application filing requirements-nonprovisional application).

[ ] continuation-in-part (C-I-P).

INVENTORSHIP IDENTIFICATION

WARNING: *If the inventors are each not the inventors of all the claims, an explanation of the facts, including the ownership of all the claims at the time the last claimed invention was made, should be submitted*

My residence, post office address and citizenship are as stated below, next to my name. I believe that I am the original, first and sole inventor *(if only one name is listed below)* or an original, first and joint inventor *(if plural names are listed below)* of the subject matter that is claimed, and for which a patent is sought on the invention entitled:

TITLE OF INVENTION

USE OF INTERFERON ALPHA 5 IN THE TREATMENT OF VIRAL LIVER DISEASES

SPECIFICATION IDENTIFICATION

The specification of which:

(complete (a), (b), or (c))

(a) [ ] is attached hereto.

NOTE: *"The following combinations of information supplied in an oath or declaration filed on the application filing date with a specification are acceptable as minimums for identifying a specification and compliance with any one of the items below will be accepted as complying with the identification requirement of 37 C.F.R. Section 1.63:*

"(1) name of inventor(s), and reference to an attached specification which is both attached to the oath or declaration at the time of execution and submitted with the oath or declaration on filing;

"(2) name of inventor(s), and attorney docket number which was on the specification as filed; or

"(3) name of inventor(s), and title which was on the specification as filed."

Notice of July 13, 1995 (1177 O.G. 60).

(b) [ ] was filed on _____, [ ] as Application No. _____
[ ] and was amended on _____ (if applicable).

NOTE: *Amendments filed after the original papers are deposited with the PTO that contain new matter are not accorded a filing date by being referred to in the declaration. Accordingly, the amendments involved are those filed with the application papers or, in the case of a supplemental declaration, are those amendments claiming matter not encompassed in the original statement of invention or claims. See 37 C.F.R. Section 1.67.*

NOTE: *"The following combinations of information supplied in an oath or declaration filed after the filing date are acceptable as minimums for identifying a specification and compliance with any one of the items below will be accepted as complying with the identification requirement of 37 C.F.R. Section 1.63:*

(A) application number (consisting of the series code and the serial number, e.g., 08/123,456);
(B) serial number and filing date;
(C) attorney docket number which was on the specification as filed;
(D) title which was on the specification as filed and reference to an attached specification which is both attached to the oath or declaration at the time of execution and submitted with the oath or declaration; or
(E) title which was on the specification as filed and accompanied by a cover letter accurately identifying the application for which it was intended by either the application number (consisting of the series code and the serial number, e.g., 08/123,456), or serial number and filing date. Absent any statement(s) to the contrary, it will be presumed that the application filed in the PTO is the application which the inventor(s) executed by signing the oath or declaration

M.P.E.P. Section 601.01(a), 7th ed

(c) [ ] was described and claimed in PCT International Application No. PCT/ES99/00134 filed on 13.5.99 and as amended under PCT Article 19 on _____ *(if any)*.

SUPPLEMENTAL DECLARATION (37 C.F.R. Section 1.67(b))

(complete the following where a supplemental declaration is being submitted)

[ ] I hereby declare that the subject matter of the

[ ] attached amendment
[ ] amendment filed on _____.

was part of my/our invention and was invented before the filing date of the original application, above identified, for such invention.

ACKNOWLEDGMENT OF REVIEW OF PAPERS AND DUTY OF CANDOR

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information, which is material to patentability as defined in 37, Code of Federal Regulations, Section 1.56,

(also check the following items, if desired)

[ ] and which is material to the examination of this application, namely, information where there is a substantial likelihood that a reasonable Examiner would consider it important in deciding whether to allow the application to issue as a patent, and

[ ] in compliance with this duty, there is attached an information disclosure statement, in accordance with 37 C.F.R. Section 1.98.

PRIORITY CLAIM (35 U.S.C. Section 119(a)-(d))

NOTE: "The claim to priority need be in no special form and may be made by the attorney or agent if the foreign application is referred to in the oath or declaration as required by Section 1.63. The claim for priority and the certified copy of the foreign application specified in 35 U.S.C. Section 119(b) must be filed in the case of an interference (Section 1.630), when necessary to overcome the date of a reference relied upon by the examiner, when specifically required by the examiner, and in all other situations, before the patent is granted. If the claim for priority or the certified copy of the foreign application is filed after the date the issue fee is paid, it must be accompanied by a petition requesting entry and by the fee set forth in Section 1.17(i). If the certified copy is not in the English language, a translation need not be filed except in the case of interference; or when necessary to overcome the date of a reference relied upon by the examiner; or when specifically required by the examiner, in which event an English language translation must be filed together with a statement that the translation of the certified copy is accurate." 37 C.F.R. Section 1.55(a).

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

(complete (d) or (e))

(d) [ ] no such applications have been filed.
(e) [X] such applications have been filed as follows.

NOTE: Where item (c) is entered above and the International Application which designated the U.S itself claimed priority check item (e), enter the details below and make the priority claim.

PRIOR FOREIGN/PCT APPLICATION(S) FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS APPLICATION AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. SECTION 119(a)-(d)

COUNTRY (OR INDICATE IF PCT)	APPLICATION NUMBER	DATE OF FILING DAY, **MONTH**, YEAR	PRIORITY CLAIMED UNDER 35 USC 119
ES	P9801003	13 May 1998	[X]YES [ ]NO
			[ ]YES [ ]NO
			[ ]YES [ ]NO
			[ ]YES [ ]NO
			[ ]YES [ ]NO

CLAIM FOR BENEFIT OF PRIOR U.S. PROVISIONAL APPLICATION(S)
(35 U.S.C. Section 119(e))

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below:

PROVISIONAL APPLICATION NUMBER	**FILING DATE**
_____/_____	_____
_____/_____	_____
_____/_____	_____

CLAIM FOR BENEFIT OF EARLIER U.S./PCT APPLICATION(S) UNDER 35 U.S.C. SECTION 120

[ ] The claim for the benefit of any such applications are set forth in the attached ADDED PAGES TO COMBINED DECLARATION AND POWER OF ATTORNEY FOR DIVISIONAL, CONTINUATION OR CONTINUATION-IN-PART (C-I-P) APPLICATION.

ALL FOREIGN APPLICATION(S), *IF ANY*, FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

_____

_____

_____

NOTE: If the application filed more than 12 months from the filing date of this application is a PCT filing forming the basis for this application entering the United States as (1) the national stage, or (2) a continuation, divisional, or continuation-in-part, then also complete ADDED PAGES TO COMBINED DECLARATION AND POWER OF ATTORNEY FOR DIVISIONAL, CONTINUATION OR C-I-P APPLICATION for benefit of the prior U.S. or PCT application(s) under 35 U.S.C. Section 120.

POWER OF ATTORNEY

I hereby appoint the following practitioner(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

(list name and registration number)

JOSEPH H. HANDELMAN, 26179	RICHARD P. BERG, 28145
JOHN RICHARDS, 31053	JULIAN H. COHEN, 20302
RICHARD J. STREIT, 25765	WILLIAM R. EVANS 25858
PETER D. GALLOWAY, 27885	JANET I. CORD, 33778
IAN C. BAILLIE, 24090	CLIFFORD J. MASS, 30086
THOMAS F. PETERSON, 24790	CYNTHIA R. MILLER, 34678

Customer # 00140

(Check the following item, if applicable)

[ ] I hereby appoint the practitioner(s) associated with the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

[ ] Attached, as part of this declaration and power of attorney, is the authorization of the above-named practitioner(s) to accept and follow instructions from my representative(s).

NOTE: "Special care should be taken in continuation or divisional applications to ensure that any change of correspondence address in a prior application is reflected in the continuation or divisional application. For example, where a copy of the oath or declaration from the prior application is submitted for a continuation or divisional application filed under 37 CFR 1.53(b) and the copy of the oath or declaration from the prior application designates an old correspondence address, the Office may not recognize, in the continuation or divisional application, the change of correspondence address made during the prosecution of the prior application. Applicant is required to identify the change of correspondence address in the continuation or divisional application to ensure that communications from the Office are mailed to the current correspondence address 37 CFR 1.63(d)(4) " Section 601.03, M.P.E.P., 7th Ed

SEND CORRESPONDENCE TO

Ladas & Parry
26 West 61st Street
New York, N.Y. 10023

DIRECT TELEPHONE CALLS TO:
(Name and telephone number)

(complete the following if applicable)

Since this filing is a [ ] continuation [ ] divisional there is attached hereto a Change of Correspondence Address so that there will be no question as to where the PTO should direct all correspondence.

DECLARATION

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

SIGNATURE(S)

NOTE: Carefully indicate the family (or last) name, as it should appear on the filing receipt and all other document.

NOTE: Each inventor must be identified by full name, including the family name, and at least one given name without abbreviation together with any other given name or initial, and by his/her residence, post office address and country of citizenship. 37 C.F.R. Section 1 63(a)(3).

NOTE: Inventors may execute separate declarations/oaths provided each declaration/oath sets forth all the inventors. Section 1.63(a)(3) requires that a declaration/oath, inter alia, identify each inventor and prohibits the execution of separate declarations/oaths which each sets forth only the name of the executing inventor 62 Fed. Reg. 53,131, 53,142, October 10, 1997.

Full name of sole or first inventor

1-00

Jesús — *(Given Name)* | *(Middle Initial or Name)* | PRIETO VALTUEÑA — *Family (Or Last Name)*

Inventor's signature Jesus Prieto Valtueña

Date 25-10-00 **Country of Citizenship** SPAIN

Residence C/ Tudela, 22 - 4º - Pamplona, Navarra, Spain

Post Office Address Same as above ES

Full name of second joint inventor, if any

2-00

Mª Pilar — *(Given Name)* | *(Middle Initial or Name)* | CIVEIRA MURILLO — *Family (Or Last Name)*

Inventor's signature Mª Pilar Civeira Murillo

Date 26-10-00 **Country of Citizenship** SPAIN

Residence C/ Irunlarrea, 35 - 1º, Pamplona, Navarra, Spain

Post Office Address Same as above ES

Full name of third joint inventor, if any

3-00

Esther — *(Given Name)* | *(Middle Initial or Name)* | LARREA LEOZ — *Family (Or Last Name)*

Inventor's signature Esther Larrea Leoz

Date 24-10-00 **Country of Citizenship** SPAIN

Residence Avda. Sancho el Fuerte, 34 - 3º C, Pamplona, Navarra, Spain

Post Office Address Same as above ES

(check proper box(es) for any of the following added page(s) that form a part of this declaration)

[ ] **Signature** for fourth and subsequent joint inventors. *Number of pages added* _____

* * *

[ ] **Signature** by administrator(trix), executor(trix) or legal representative for deceased or incapacitated inventor. *Number of pages added* _____

* * *

[ ] **Signature** for inventor who refuses to sign or cannot be reached by person authorized under 37 C.F.R. Section 1.47. *Number of pages added* _____

* * *

[ ] Added page for **signature** by one joint inventor on behalf of deceased inventor(s) where legal representative cannot be appointed in time. (37 C.F.R. Section 1.47)

* * *

[ ] Added pages to combined declaration and power of attorney for divisional, continuation, or continuation-in-part (C-I-P) application.

[ ] Number of pages added _____

* * *

[ ] Authorization of practitioner(s) to accept and follow instructions from representative.

(If no further pages form a part of this Declaration, then end this Declaration with this page and check the following item)

[ ] This declaration ends with this page.

V. SIGNATURES

(complete only (e) or (f) below)

(e)
NOTE: All inventors must sign the statement.

_____
Name of Inventor

_____ Date: _____
Signature of Inventor

_____
Name of Inventor

_____ Date: _____
Signature of Inventor

_____
Name of Inventor

_____ Date: _____
Signature of Inventor

(add lines for any additional inventors who must sign)

or

(f)
NOTE: The title of the person signing on behalf of a concern or nonprofit organization should be specified.

Name of Person Signing _____

Title of Person _____
(if signing on behalf of a concern or non-profit organization)

Address of Person Signing INSTITUTO CIENTIFICO Y TECNOLOGICO DE NAVARRA, S.A.
Avda. Pío XII, 53 - 31008 Pamplona, Navarra, Spain

SIGNATURE Fernando de la Puente García Sanjes DATE 24/10/00

Practitioner's Docket No. U013039-2 *PATENT*

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

[X] In re application of: Jesús PRIETO VALTUEÑA, et al.
Application No.: PCT/ES99/00134 Group No.:
Filed: 13 May 1999 Examiner:
For: "USE OF INTERFERON ALPHA 5 IN THE TREATMENT OF VIRAL LIVER DISEASES"
[ ] *Patent No.: Issue Date:

**NOTE. Insert name(s) of inventor(s) and title also for patent Where statement is with respect to a maintenance fee payment, also insert application number and filing date, and add Box M. Fee to address.*

STATEMENT CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(c-f) and 1.27(b-d))

With respect to the invention described in
[ ] the specification filed herewith.
[X] application no. PCT/ES99/00134 filed 13 May 1999 .
[ ] patent no. _____ issued _____.

I. IDENTIFICATION AND RIGHTS AS A SMALL ENTITY

I hereby state that I am

(complete either (a), (b), (c) or (d) below)

(a) Independent Inventor
[ ] a below named independent inventor, and that I qualify as an independent inventor, as defined in 37 CFR 1.9(c), for purposes of paying reduced fees under Sections 41(a) and (b) of Title 35, United States Code, to the Patent and Trademark Office.

(b) Noninventor Supporting a Claim by Another
[ ] making this statement to support a claim by

_____

for a small entity status for purposes of paying reduced fees under Sections 41(a) and (b) of Title 35, United States Code. I hereby state that I would qualify as an independent inventor as defined in 37 CFR 1.9(c) for purposes of paying reduced fees under Sections 41(a) and (b) of Title 35, United States Code, if I had made the above identified invention.

(c) Small Business Concern
[ ] the owner of the small business concern identified below:
check one → [X] an official of the small business concern empowered to act on behalf of the concern identified below:

Name of Concern INSTITUTO CIENTIFICO Y TECNOLOGICO DE NAVARRA, S.A.
Address of Concern Avda. Pío XII, 53 - 31008 Pamplona, Navarra, Spain
and

that the above identified small business concern qualifies as a small business concern, as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under Sections 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

(d) Non-Profit Organization

[ ] an official empowered to act on behalf of the nonprofit organization identified below:

Name of Organization _____
Address of Organization _____

TYPE OF ORGANIZATION

[ ] University or Other Institution of Higher Education

[ ] Tax Exempt Under Internal Revenue Service Code (26 USC 501(a) and 501(c) (3))

[ ] Nonprofit Scientific or Educational Under Statute of State of the United States of America
(Name of State_____)
(Citation of Statute_____)

[ ] Would Qualify as Tax Exempt Under Internal Revenue Service Code (26 USC 501(a) and 501(c) (3)), if Located in the United States of America

[ ] Would Qualify as Nonprofit Scientific or Educational Under Statute of State of the United States of America, if Located in the United States of America
(Name of State_____)
(Citation of Statute_____)

and that the nonprofit organization identified above qualifies as a nonprofit organization, as defined in 37 CFR 1.9(e), for purposes of paying reduced fees under Sections 41(a) and (b) of Title 35, United States Code.

II. OWNERSHIP OF INVENTION BY DECLARANT

I hereby state that rights under contract or law remain with and/or have been conveyed to the above identified

[ ] person	[x] concern	[ ] organization
(item (a) or (b) above)	(item (c) above)	(item (d) above)

EXCEPT, that if the rights held are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held (1) by any person who could not be classified as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, (2) any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or (3) a nonprofit organization under 37 CFR 1.9(e).

[X] no such person, concern, or organization
[ ] person, concerns or organizations listed below*

*NOTE: *Separate statements are required from each named person, concern or organization having rights to the invention as to their status as small entities. (37 CFR 1.27)*

Full Name _____
Address _____
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

Full Name _____
Address _____
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

III. ACKNOWLEDGEMENT OF DUTY TO NOTIFY PTO OF STATUS CHANGE

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

IV. DECLARATION

(check the following item, if desired)

NOTE: The following verification statement need not be made in accordance with the rules published on October 10, 1997, 62 Fed. Reg. 52131, effective December 1, 1997.

NOTE: "The presentation to the Office (whether by signing, filing, submitting, or later advocating) of any paper by a party, whether a practitioner or non-practitioner, constitutes a certification under § 10.18(b) of this chapter. Violations of § 10.18(b)(2) of this chapter by a party, whether a practitioner or non-practitioner, may result in the imposition of sanctions under § 10.18(c) of this chapter. Any practitioner violating § 10.18(b) may also be subject to disciplinary action. See §§ 10.18(d) and 10.23(c)(15)." 37 CFR 1.4(d)(2).

[ ] I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NO:8) was used to determine genotype 4.

Pages 27 - 29 delete in entirety and substitute the accompanying Sequence Listing:

SMD Pct/09

RAW SEQUENCE LISTING
PATENT APPLICATION: US/09/674,445

DATE: 10/16/2001
TIME: 08:44:12

Input Set : A:\PTO.txt
Output Set: N:\CRF3\10162001\I674445.raw

ENTERED

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      3 <110> APPLICANT: Prieto Valtuena, Jesus
      4       Civeira Murillo, Pilar
      5       Larrea Leoz, Esther
      7 <120> TITLE OF INVENTION: USE OF INTERFERON ALPHA 5 IN THE TREATMENT OF VIRAL LIVER
DISEASES
      9 <130> FILE REFERENCE: U-013039-2
     11 <140> CURRENT APPLICATION NUMBER: 09/674445
C--> 13 <141> CURRENT FILING DATE: 2001-07-05
     15 <150> PRIOR APPLICATION NUMBER: PCT ES99/00134
     17 <151> PRIOR FILING DATE: 1999-05-13
     19 <150> PRIOR APPLICATION NUMBER: ES P9801003
     21 <151> PRIOR FILING DATE: 1998-05-13
     23 <160> NUMBER OF SEQ ID NOS: 8
     25 <210> SEQ ID NO: 1
     27 <211> LENGTH: 274
     29 <212> TYPE: DNA
     31 <213> ORGANISM: Homo sapien
     33 <220> FEATURE:
     35 <223> OTHER INFORMATION: Nucleotides 672 - 945 in the sequence of the IFNa5 gene
published in
     36       the Genbank database under access number X02956.
     38 <400> SEQUENCE: 1
     40 tc cat gag atg atc cag cag acc ttc aat ctc ttc agc aca aag gac tca      50
     41 His Glu Met Ile Gln Gln Thr Phe Asn Leu Phe Ser Thr Lys Asp Ser
     42 1               5                  10                  15
     44 tct gct act tgg gat gag aca ctt cta gac aaa ttc tac act gaa ctt tac    101
     45 Ser Ala Thr Trp Asp Glu Thr Leu Leu Asp Lys Phe Tyr Thr Glu Leu Tyr
     46             20                  25                  30
     48 cag cag ctg aat gac ctg gaa gcc tgt atg atg cag gag gtt gga gtg gaa    152
     49 Gln Gln Leu Asn Asp Leu Glu Ala Cys Met Met Gln Glu Val Gly Val Glu
     50         35                  40                  45                  50
     52 gac act cct ctg atg aat gtg gac tct atc ctg act gtg aga aaa tac ttt    203
     53 Asp Thr Pro Leu Met Asn Val Asp Ser Ile Leu Thr Val Arg Lys Tyr Phe
     54                 55                  60                  65
     56 caa aga atc acc ctc tat ctg aca gag aag aaa tac agc cct tgt gca tgg    254
     57 Gln Arg Ile Thr Leu Tyr Leu Thr Glu Lys Lys Tyr Ser Pro Cys Ala Trp
     58           70                  75                  80
     59 gag gtt gtc aga gca gaa at                                          274
     60 Glu Val Val Arg Ala Glu
     61 85                  90
     64 <210> SEQ ID NO: 2
     66 <211> LENGTH: 20
     68 <212> TYPE: DNA
     70 <213> ORGANISM: Artificial Sequence
     72 <220> FEATURE:
     74 <223> OTHER INFORMATION: Primer to amplify a fragment of 274 base pairs located
     75       between nucleotides 240 - 514 in the human IFNa gene
     77 <400> SEQUENCE: 2
     79 tccatgagat gatccagcag                                                20
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RAW SEQUENCE LISTING DATE: 10/16/2001
PATENT APPLICATION: US/09/674,445 TIME: 08:44:12

Input Set : A:\PTO.txt
Output Set: N:\CRF3\10162001\I674445.raw

178 <220> FEATURE:
180 <223> OTHER INFORMATION: DNA fragment for use in determining C virus genotype
182 <400> SEQUENCE: 8
184 grccgtcttg gggccmaaat gat 23

VERIFICATION SUMMARY
PATENT APPLICATION: US/09/674,445

DATE: 10/16/2001
TIME: 08:44:13

Input Set : A:\PTO.txt
Output Set: N:\CRF3\10162001\I674445.raw

L:13 M:271 C: Current Filing Date differs, Replaced Current Filing Date

SEQUENCE LISTING

<110> Prieto Valtuena, Jesus
Civeira Murillo, Pilar
Larrea Leoz, Esther

<120> USE OF INTERFERON ALPHA 5 IN THE TREATMENT OF VIRAL LIVER DISEASES

<130> U-013039-2

<140> 09/674445

<141> 2000-11-01

<150> PCT ES99/00134

<151> 1999-05-13

<150> ES P9801003

<151> 1998-05-13

<160> 8

<210> 1

<211> 274

<212> DNA

<213> Homo sapien

<220>

<223> Nucleotides 672 - 945 in the sequence of the IFNα5 gene published in the Genbank database under access number X02956.

<400> 1

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   His Glu Met Ile Gln Gln Thr Phe Asn Leu Phe Ser Thr Lys Asp Ser
     1               5                  10                  15

tct gct act tgg gat gag aca ctt cta gac aaa ttc tac act gaa ctt tac    101
Ser Ala Thr Trp Asp Glu Thr Leu Leu Asp Lys Phe Tyr Thr Glu Leu Tyr
             20                  25                  30

cag cag ctg aat gac ctg gaa gcc tgt atg atg cag gag gtt gga gtg gaa    152
Gln Gln Leu Asn Asp Leu Glu Ala Cys Met Met Gln Glu Val Gly Val Glu
     35                  40                  45                  50

gac act cct ctg atg aat gtg gac tct atc ctg act gtg aga aaa tac ttt    203
Asp Thr Pro Leu Met Asn Val Asp Ser Ile Leu Thr Val Arg Lys Tyr Phe
                 55                  60                  65
```

caa aga atc acc ctc tat ctg aca gag aag aaa tac agc cct tgt gca tgg 254
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70 75 80

gag gtt gtc aga gca gaa at 274
Glu Val Val Arg Ala Glu
85 90

<210> 2

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer to amplify a fragment of 274 base pairs located between nucleotides 240 - 514 in the human IFNα gene

<400> 2

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<210> 3

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer to amplify a fragment of 274 base pairs located between nucleotides 240 - 514 in the human IFNα gene

<400> 3

atttctgctc tgacaacctc cc 22

<210> 4

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer to amplify a fragment of 276 base pairs located between nucleotides 349 - 625 of cDNA of human IFNβ gene

<400> 4

tctagcactg gctggaatga g 21

<210> 5

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer to amplify a fragment of 276 base pairs located between nucleotides 349 - 625 of cDNA of human IFNβ gene

<400> 5

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<210> 6

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer to amplify a fragment of 314 base pairs (nucleotides 1319 - 2079) of the β-actin gene

<400> 6

tctacaatga gctgcgtgtg 20

<210> 7

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer to amplify a fragment of 314 base pairs

(nucleotides 1319 - 2079) of the β-actin gene

<400> 7

ggtgaggatc ttcatgaggt 20

<210> 8

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> DNA fragment for use in determining C virus genotype

<400> 8

grccgtcttg gggccmaaat gat 23

REMARKS

The above amendatory action is taken in response to the Official Action of May 4, 2001 and satisfies the requirements of 37 CFR 1.821 - 1.825. A computer readable form copy of the Sequence Listing and the requisite statements are submitted herewith. Also submitted herewith is a marked-up copy of the amended paragraphs of the specification to locate the amendments.

Respectfully submitted,

CLIFFORD J. MASS
LADAS & PARRY
26 WEST 61ST STREET
NEW YORK, NY 10023
REG. NO. 30,086 (212) 708-1890

c:\docs\13039pre.jul

RNAm levels of IFNα and IFNβ were determined using a quantitative polymerase chain reaction reverse transcription (RT-PCR) method using a thermocycler (Perkin-Elmer Gene Amp PCR system 2400). Prior to reverse transcription 2 μg of total RNA (from both the liver and PBMC) were treated with 1 unit of deoxyribonuclease (DNAse I amplification grade, Gibco-BRL, Gaithersburg, MD, USA) to eliminate possible contaminating DNA. The presence of traces of DNA was checked by including control reactions without reverse transcription. This step is required because of the absence of introns in IFNα and IFNβ genes (18), which made it impossible for us to distinguish the product of PCR from the RNA or possible contaminating DNA. All the controls performed without reverse transcription were negative, indicating the absence of contaminating DNA. Total RNA was transcribed (60 minutes at 37°C) with 400 units of M-MuLV reverse transcriptase (Gibco- BRL, Gaithersburg, MD, USA) in a final volume of 40 μl of 5 x saline solution (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), supplemented with 5 mM DTT, 0.5 mM triphosphate dioxyribonucleotides (Boehringer Mannheim, Mannheim, Germany), 48 units of RNAsas inhibitor (Promega Corporation, MD, US) and 400 ng of random hexamers (Boehringer Mannheim, Mannheim, Germany). After denaturing the reverse transcriptase (95°C, 1 minute) and rapidly cooling over ice, a 10 μl aliquot (0.5 μg) of the cDNA was used to amplify the IFNα and IFNβ by PCR in 50 μl of 10 x PCR buffer (160 mM $(NH_4)SO_4$, 670 mM Tris-HCl pH 8.8, 0.1% Tween 20) supplemented with the direction and antidirection primers (40 ng of each one for IFNα and 60 ng for IFNβ), 1.2 mM $MgCl_2$ and 2 units of Biotaq™ DNA polymerase (Bioline, London, LTK). Control reactions without RNA were performed in all the experiments. As an internal control for each sample a

fragment of β-actin cDNA was amplified using a 10 µl aliquot of the cDNA obtained previously. The IFNα was amplified by performing 30 or 33 cycles (PBMC or liver respectively) (94°C, 60°C and 72°C during 20, 15 and 30 seconds for each step respectively), the INFβ was amplified by performing 30 or 35 cycles (PBMC or liver respectively) (94°C, 58°C and 72°C for 20, 15 and 30 seconds for each step respectively) and β-actin was amplified by reacting 18 or 25 cycles (PBMC or liver respectively) (94°C, 55°C and 72°C for 20, 15 and 30 seconds for each step respectively), protocols which avoid interference with the PCR reaction saturation stage. The oligonucleotides (5'-3') d(TCCATGAGATGATCCAGCAG) (SEQ ID NO:2) and d(ATTTCTGCTCTGACAACCTCCC) (SEQ ID NO:3) were used as direction and antidirection primers respectively to amplify a fragment of 274 pairs of bases located between nucleotides 240-514 in the human IFNα gene (19). These oligonucleotides are direction primers designed to amplify all the subtypes of IFNα. The oligonucleotides D(TCTAGCACTGGCTGGAATGAG) (SEQ ID NO:4) and d(GTTTCGGAGGTAACCTGTAAG) (SEQ ID NO:5) were the primers used to amplify a fragment of 276 base pairs located between nucleotides 349-625 of cDNA of human IFNβ (20) d(TCTACAATGAGCTGCGTGTG) (SEQ ID NO:6) and d(GGTGAGGATCTTCATGAGGT) (SEQ ID NO:7) were the primers used to amplify a fragment of 314 base pairs (nucleotides 1319-2079) of the β-actin gene (21).

Please rewrite the paragraph bridging pages 12 - 13 as follows:

The presence of C virus RNA in serum was determined using the RT-PCR technique (14, 22), using 2 pairs of specific primers for the non-coding 5' region of the C virus genome. The C virus RNA was quantified using the competitive PCR technique previously described by

ourselves (22). The viral genotype was determined using Viazov's method (23) as already described previously (22, 24). The test 5'G~~(A,G)~~(R)CCGTCTTGGGGCC~~(A,C)~~(M)AAATGAT (SEQ ID NO:8) was used to determine genotype 4.

SEQUENCE LISTING

<110> Prieto Valtuena, Jesus
Civeira Murillo, Pilar
Larrea Leoz, Esther

<120> USE OF INTERFERON ALPHA 5 IN THE TREATMENT OF VIRAL LIVER DISEASES

<130> U-013039-2

<140> 09/674445

<141> 2000-11-01

<150> PCT ES99/00134

<151> 1999-05-13

<150> ES P9801003

<151> 1998-05-13

<160> 8

<210> 1

<211> 274

<212> DNA

<213> Homo sapien

<220>

<223> Nucleotides 672 - 945 in the sequence of the IFNα5 gene published in the Genbank database under access number X02956.

<400> 1

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tct gct act tgg gat gag aca ctt cta gac aaa ttc tac act gaa ctt tac    101
Ser Ala Thr Trp Asp Glu Thr Leu Leu Asp Lys Phe Tyr Thr Glu Leu Tyr
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Gln Gln Leu Asn Asp Leu Glu Ala Cys Met Met Gln Glu Val Gly Val Glu
     35                  40                  45                  50

gac act cct ctg atg aat gtg gac tct atc ctg act gtg aga aaa tac ttt    203
Asp Thr Pro Leu Met Asn Val Asp Ser Ile Leu Thr Val Arg Lys Tyr Phe
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caa aga atc acc ctc tat ctg aca gag aag aaa tac agc cct tgt gca tgg    254
Gln Arg Ile Thr Leu Tyr Leu Thr Glu Lys Lys Tyr Ser Pro Cys Ala Trp
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<212> DNA

<213> Artificial Sequence

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<223> Primer to amplify a fragment of 274 base pairs located between nucleotides 240 - 514 in the human IFNα gene

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<223> Primer to amplify a fragment of 276 base pairs located between nucleotides 349 - 625 of cDNA of human IFNβ gene

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<223> Primer to amplify a fragment of 314 base pairs (nucleotides 1319 - 2079) of the β-actin gene

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<223> DNA fragment for use in determining C virus genotype

<400> 8

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LIST OF SEQUENCES

GENERAL INFORMATION:

APPLICANT:

NAME: INSTITUTO CIENTIFICO Y TECNOLOGICO
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TITLE: "USE OF ALPHA 5 INTERFERON IN THE TREATMENT OF VIRAL LIVER DISEASES".

NUMBER OF SEQUENCES: 1

ADDRESS FOR CORRESPONDENCE:

NAME: INSTITUTO CIENTIFICO Y TECNOLOGICO
DE NAVARRA, S.A.
STREET: Avenida Pío XII, 53
TOWN: Pamplona
STATE OR PROVINCE: Navarra
COUNTRY: Spain
POST CODE: 31008

COMPUTER READABLE FORM:

TYPE OF MEDIUM: 3.5" DISK

COMPUTER: PC
OPERATING SYSTEM: WINDOWS
WORD PROCESSOR: WORD

INFORMATION ON THE LAWYER/AGENT:
NAME: ALBERTO DE ELZABURU
REGISTER NUMBER: 232/1
REFERENCE/REGISTER NUMBER: P-99043

TELECOMMUNICATIONS INFORMATION:
TELEPHONE: 91 7009400
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TELEX OR ELECTRONIC MAIL: elzaburu@elzaburu.es

INFORMATION FOR SEQ ID NO.: 1

CHARACTERISTICS OF THE SEQUENCE:
LENGTH: 274 base pairs
TYPE: nucleotides
NUMBER OF STRANDS: 1
CONFIGURATION: linear
TYPE OF MOLECULE: cDNA
HYPOTHETICAL: NO
ANTI-DIRECTION: NO
SOURCE OF ORIGIN:
Organism: Homo Sapiens
Type of tissue: liver
POSITION IN THE GENOME: Chromosome 9
CHARACTERISTIC:
NAME/KEY: IFNα5
Method of identification: RT-PCR, Sequencing
OTHER INFORMATION:

Nucleotides 672 to 945 in the sequence of the IFNα5 gene published in the Genbank database under access number X02956.

DESCRIPTION OF THE SEQUENCE SEQ ID NO: 1

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TC CAT GAG ATG ATC CAG CAG ACC TTC AAT CTC TTC AGC ACA AAG GAC TCA      50
   His Glu Met Ile Gln Gln Thr Phe Asn Leu Phe Ser Thr Lys Asp Ser
    1               5                  10                  15
TCT GCT ACT TGG GAT GAG ACA CTT CTA GAC AAA TTC TAC ACT GAA CTT TAC    101
Ser Ala Thr Trp Asp Glu Thr Leu Leu Asp Lys Phe Tyr Thr Glu Leu Tyr
            20                  25                  30
CAG CAG CTG AAT GAC CTG GAA GCC TGT ATG ATG CAG GAG GTT GGA GTG GAA    152
Gln Gln Leu Asn Asp Leu Glu Ala Cys Met Met Gln Glu Val Gly Val Glu
    35                  40                  45                  50
GAC ACT CCT CTG ATG AAT GTG GAC TCT ATC CTG ACT GTG AGA AAA TAC TTT    203
Asp Thr Pro Leu Met Asn Val Asp Ser Ile Leu Thr Val Arg Lys Tyr Phe
                55                  60                  65
CAA AGA ATC ACC CTC TAT CTG ACA GAG AAG AAA TAC AGC CCT TGT GCA TGG    254
Gln Arg Ile Thr Leu Tyr Leu Thr Glu Lys Lys Tyr Ser Pro Cys Ala Trp
        70                  75                  80
GAG GTT GTC AGA GCA GAA AT                                            274
Glu Val Val Arg Ala Glu
85                  90
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